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Comparison of the Luminex xTAG Respiratory Viral Panel with xTAG Respiratory Viral Panel Fast for Diagnosis of Respiratory Virus Infections[▽]

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Nucleic acid tests are sensitive and specific and provide a rapid diagnosis, making them invaluable for patient and outbreak management. Multiplex PCR assays have additional advantages in providing an economical and comprehensive panel for many common respiratory viruses. Previous reports have shown the utility of the xTAG respiratory viral panel (RVP) assay manufactured by Luminex Molecular Diagnostics for this purpose. A newer generation of this kit, released in Canada in early 2010, is designed to simplify the procedure and reduce the turnaround time by about 24 h. The assay methodology and targets included in this version of the kit are different; consequently, the objective of this study was to compare the detection of a panel of respiratory viral targets using the older Luminex xTAG RVP (RVP Classic) assay with that using the newer xTAG RVP Fast assay. This study included 334 respiratory specimens that had been characterized for a variety of respiratory viral targets; all samples were tested by both versions of the RVP assay in parallel. Overall, the RVP Classic assay was more sensitive than the RVP Fast assay (88.6% and 77.5% sensitivities, respectively) for all the viral targets combined. Targets not detected by the RVP Fast assay included primarily influenza B virus, parainfluenza virus type 2, and human coronavirus 229E. A small number of samples positive for influenza A virus, respiratory syncytial virus B, human metapneumovirus, and parainfluenza virus type 1 were not detected by the RVP Classic assay and in general had low viral loads.

The utility of the xTAG respiratory viral panel (RVP) (RVP Classic) assay, manufactured by Luminex Molecular Diagnostics for the detection of a panel of respiratory viruses, has been demonstrated. Previous reports have shown this assay to have comparable or superior sensitivity compared to those of direct fluorescent-antibody assay (DFA)-, culture-, and PCR-based methods (10, 14, 20). The main drawbacks of this assay were its lengthy protocol, resulting in longer turnaround times, and the need for a manipulation of the amplified product, which could be a potential clinical laboratory contamination risk. The RVP Classic assay was modified to have a simpler protocol, resulting in a shorter turnaround time, and was marketed as the xTAG RVP Fast assay. The targets detected have been slightly altered to allow the detection of influenza A virus (IFVA), with additional subtyping of positive specimens into subtypes H1 and H3; influenza B virus (IFVB); respiratory syncytial virus (RSV) types 1 and 2; human coronaviruses (hCoVs) NL63, 229E, OC43, and HKU1; parainfluenza viruses (PIV) types 1 to 4; human metapneumovirus (hMPV); picornaviruses (including enteroviruses [EV] and rhinoviruses [RVs]); and a range of adenovirus (ADV) types. This version of the kit can additionally detect the presence of human bocavirus (hBoV). In addition, RNA bacteriophage MS2 is used as an internal extraction/inhibition control, and DNA bacteriophage lambda is used as an amplification and assay performance control.

The RVP Fast assay incorporates multiplex reverse transcriptase PCR (RT-PCR) using primers with proprietary universal tags. The amplified product is hybridized to a bead array conjugated to specific probes, and detection is performed using a streptavidin-R-phycoerythrin conjugate. Each Luminex bead population detects a unique viral target by hybridization to a specific anti-tag. A signal (median fluorescence intensity [MFI]) is generated for each bead population. These fluorescence values are analyzed by using xTAG data analysis software for RVP Fast (TDAS RVP Fast) to establish the presence or absence of viral targets in each sample. A single multiplex reaction thus identifies all 19 viral targets.

Multiplexed array approaches have the benefit of broad viral detection in a short time using a limited amount of sample compared to traditional methods. The use of multiplex technologies for the detection of respiratory targets was previously reported (1, 2, 3, 12, 13, 14, 15, 16, 17). Here we report the results of a study to evaluate the performance of the RVP Fast assay, which was recently released in Canada, compared to that of the older version, the RVP Classic assay.

MATERIALS AND METHODS

Clinical specimens. Respiratory specimens (n = 334) submitted to the Provincial Laboratory for Public Health (ProvLab), Alberta, Canada, were included in this study. According to the diagnostic algorithm for the testing of respiratory specimens at the ProvLab, all samples were prescreened for influenza A virus (IFVA) and influenza B virus (IFVB) by real-time RT-PCR (18). The IFVA-positive samples were subtyped by using methods described previously (18, 21). Samples that tested negative for IFVA and IFVB were tested by the RVP Classic assay according to the manufacturer's instructions. This study included positive and negative samples detected by the above-described testing algorithm.

Of the 334 samples tested, 291 were positive and 43 were retrospectively

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negative using the testing algorithm described above based upon which samples were included in this study. The majority of specimens tested were nasopharyngeal (NP) swabs or aspirates (n = 243; 72.75%), nasal swabs (NSs) (n = 13; 3.89%), throat swabs (TSs) (n = 36; 10.78%), bronchoalveolar lavage (BAL) fluid (n = 18; 5.39%), sputum (n = 7; 2.10%), and fluids/swabs of unknown respiratory origin (n = 17; 5.09%).

The patient age range for the study samples was distributed from 11 days to 91 years, with a median of 6 years. The most represented group (42.5%) was infants and children less than 2 years of age.

Study design. Frozen extracts were used in this study; however, testing by both the RVP Classic and RVP Fast assays was performed concurrently to alleviate any problems relating to sample degradation or sporadic weakly positive results. The data presented here are based on results obtained by concurrent testing with both assays.

Samples with discordant results were further confirmed by using a combination of published and in-house-validated unpublished real-time PCR and RT-PCR assays using hydrolysis probes with the 7500 SDS platform (Applied Biosystems [ABI], CA) or by nucleic acid sequence-based amplification (NASBA). The assays used were IFVA and IFVB detection (18); subtyping into subtypes H1 and H3 and the pandemic (H1N1) 2009 subtype (18, 21); RSVA and RSVB detection (22); detection of hCoVs NL63, 229E, OC43, and HKU1 (in-house [our unpublished data]); detection of PIVs 1 to 4 (8, 23) (in-house [our unpublished data]); hMPV detection (19); ADV detection (24); and hBoV detection (in-house [our unpublished data]). Picornaviruses (including enteroviruses and rhinoviruses) were confirmed by sequencing (4). Equivocal samples by the RVP Classic assay were considered positive, since all equivocal samples were confirmed as being positive by the confirmatory assays. For the RVP Fast assay, there is no designation of samples as equivocal, since the software provides only positive or negative results.

Nucleic acid extraction. Nucleic acid extraction was undertaken by using the easyMAG extractor and reagents (bioMérieux, St. Laurent, Quebec, Canada) according to the manufacturer's protocol. A specimen input volume of 200 μl and an output volume of 55 μl are suggested for the RVP Fast assay by the manufacturer; however, in all our experiments we used an input volume of 200 μl and an output volume of 110 μl. To compare the differences in target detection based on extraction volumes, 10-fold serial dilutions of IFVA, IFVB, RSVB, and ADV were extracted using both protocols, and no difference in sensitivity was observed. In addition, a subset of IFVB-positive specimens was also extracted by both protocols, and target detections were compared.

Sensitivity and reproducibility of the xTAG RVP Fast assay. Tenfold serial dilutions of nucleic acid extracts from patient specimens positive for IFVA, IFVB, RSV, and ADV were prepared in carrier RNA, and the limits of detection were compared between the RVP Classic and RVP Fast assays in replicates of 3 or 6 based on the availability of reagents. These dilutions were also tested by real-time PCR and RT-PCR assays using the confirmatory assays described above. The crossing threshold (C_T) values from these assays are semiquantitative and broadly represent the viral load in the sample.

The reproducibilities of the RVP Classic and RVP Fast assays were assessed by using samples positive for ADV, RSV, and IFVA at high and low viral loads tested in triplicate.

RESULTS

Sensitivity and reproducibility. Serial dilutions of extracts from patient specimens were concurrently tested by the RVP Classic and RVP Fast assays to compare the endpoint sensitivities for the detection of IFVA subtype H3, IFVB, ADV, and RSV. These dilutions were also tested by real-time PCR or RT-PCR assays, and C_T values for the serial dilutions are indicated in Table 1. These C_T values are semiquantitative and broadly represent the viral load in the sample. The endpoints for the detection and typing of IFVA were consistent between the two assays, with the typing assay being 10-fold less sensitive than the detection assay for both the RVP Classic and RVP Fast assays. The RVP Fast assay provided better reproducibility (6 of 6 replicates tested positive, compared to 2/6 by the RVP Classic assay) for the detection of ADV at the endpoint; however, the endpoints were comparable. The sensitivities for the detection of IFVB were comparable for the RVP Classic and RVP Fast assays for positive samples from 2008 and 2009; however, a decreased sensitivity for samples from 2010 was observed for the RVP Fast assay. The RVP Fast assay was 10-fold more sensitive for the detection of RSVA, and these results are indicated in Table 1.

TABLE 1. Comparison of sensitivities for IFVB, RSV, IFVA (subtype H3), and ADV by the RVP Classic and RVP Fast assays[a]

Target	Dilution	C_T	No. of positive replicates/total no. of replicates			
			RVP Classic		RVP Fast	
			Detection	Typing	Detection	Typing
IFVA-H3	10^{-1}	19.63	6/6	6/6	6/6	6/6
	10^{-2}	24.01	6/6	6/6	6/6	6/6
	10^{-3}	28.18	6/6	6/6	6/6	6/6
	10^{-4}	33.08	6/6	6/6	6/6	6/6
	10^{-5}	37.29	6/6	0/6	5/6	1/6
	10^{-6}	Negative	1/6	0/6	0/6	0/6
ADV	Neat	22.60	6/6		6/6	
	10^{-1}	26.01	6/6		6/6	
	10^{-2}	29.35	6/6		6/6	
	10^{-3}	32.84	2/6		6/6	
	10^{-4}	35.48	0/6		0/6	
	10^{-5}	Negative	0/6		0/6	
IFVB 2008	10^{-1}	23.34	3/3		3/3	
	10^{-2}	26.74	3/3		3/3	
	10^{-3}	30.01	3/3		3/3	
	10^{-4}	34.49	0/3		0/3	
	10^{-5}	36.60	0/3		0/3	
	10^{-6}	Negative	0/3		0/3	
IFVB 2009	10^{-1}	20.79	3/3		3/3	
	10^{-2}	24.42	3/3		3/3	
	10^{-3}	28.29	3/3		2/3	
	10^{-4}	33.20	0/3		0/3	
	10^{-5}	35.11	0/3		0/3	
	10^{-6}	Negative	0/3		0/3	
IFVB 2010	10^{-1}	18.03	3/3		3/3	
	10^{-2}	21.05	3/3		3/3	
	10^{-3}	24.59	3/3		3/3	
	10^{-4}	28.38	3/3		3/3	
	10^{-5}	31.69	3/3		0/3	
	10^{-6}	Negative	0/3		0/3	
IFVB 2010	10^{-1}	25.44	3/3		3/3	
	10^{-2}	29.24	3/3		0/3	
	10^{-3}	32.49	0/3		0/3	
	10^{-4}	35.62	0/3		0/3	
	10^{-5}	Negative	0/3		0/3	
RSVA	Neat	26.69	3/3		3/3	
	10^{-1}	29.94	3/3		3/3	
	10^{-2}	33.21	0/3		3/3	
	10^{-3}	37.18	0/3		0/3	
	10^{-4}	40.01	0/3		0/3	
	10^{-5}	Negative	0/3		0/3	

[a] Serial dilutions of patient specimens were tested in parallel by the RVP Classic and RVP Fast assays. Dilutions for IFVB and RSVA were tested in triplicate, and dilutions for IFVA and ADV were tested in replicates of 6. IFVB-positive samples from 2008, 2009, and 2010 are included.

The assay reproducibility was further tested by using a series of patient samples. Samples with high and low viral loads for ADV, RSV, and IFVA were tested by the RVP Classic and

TABLE 2. Comparison of reproducibilities for ADV, RSVA, RSVB, and IFVA by the RVP Classic and RVP Fast assays[a]

Target	C_T	No. of positive replicates/total no. of replicates			
		RVP Classic		RVP Fast	
		Detection	Typing	Detection	Typing
ADV	16	3/3		3/3	
ADV	28	3/3		3/3	
RSVB	25	3/3		3/3	
RSVA	31	2/3		3/3	
RSVB	32	3/3		3/3	
IFVA-H3	19	3/3	3/3	3/3	3/3
IFVA-H3	33	3/3	0/3	3/3	3/3
IFVA-H1	29	3/3	2/3	3/3	3/3
IFVA-H1	31	3/3	3/3	3/3	3/3
IFVA-pH1N1	21	3/3		3/3	
IFVA-pH1N1	33	3/3		3/3	

[a] Patient specimens with a variation in viral load were tested in parallel by the RVP Classic and RVP Fast assays in triplicate to assess assay reproducibility. The IFVA specimens tested were of subtypes H1 and H3 and of the pandemic (H1N1) 2009 subtype.

RVP Fast assays. The tested specimens were positive, showing the assays to provide reproducible results at the viral loads tested; a weak IFVA subtype H3 sample was detected but not subtyped by the RVP Classic assay. These results are shown in Table 2.

Summary of results for all samples. Of the 334 samples tested for this study, 291 were positive and 43 were retrospectively negative using the testing algorithm described in Materials and Methods. These samples were concurrently tested by the RVP Classic and RVP Fast assays for this study. A total of 259 samples (77.54%) gave a positive result for one or more targets, and 75 (22.45%) were negative by the RVP Fast assay. The same set of 334 samples concurrently tested by the RVP Classic assay gave a positive result for one or more targets for 296 (88.62%) samples and a negative result for 38 (11.38%) samples.

Of the 259 samples that tested positive by the RVP Fast assay, 207, 43, 8, and 1 specimens were positive for a single virus, 2 viruses, 3 viruses, and 4 viruses, respectively, for a total of 321 targets. Of the 296 samples that tested positive by the RVP Classic assay, 255, 34, and 7 specimens were positive for a single virus, 2 viruses, and 3 viruses, respectively, for a total of 344 targets. The targets not detected by the RVP Fast assay included IFVB ($n = 27$); hCoV 229E ($n = 3$), PIV2 ($n = 4$), IFVA ($n = 2$), hMPV ($n = 2$), ADV, RSVB, and PIV1 ($n = 1$ each). Three of the undetected targets were present as coinfections, including one each for IFVB, PIV2, and ADV, and the remaining 38 targets missed were single-target positive specimens. The RVP Classic assay failed to detect the following targets ($n = 11$): EV/RV ($n = 6$); hCoV OC43 ($n = 2$); and ADV, hCoV NL63, and RSVA ($n = 1$ each). Of these 11 targets not detected by the RVP Classic assay, 10 were present as coinfecting viruses, and 1 sample was positive only for EV/RV by the RVP Fast assay. This sample was originally selected as a negative sample by our testing algorithm, and the result could not be confirmed by the gel-based nested confirmatory assay (4). In addition, the RVP Fast assay detected 7 specimens that were positive for hBoV and provided subtyping for 2 additional IFVA-positive samples as subtype H3.

Of the negative samples tested, the RVP Fast assay detected EV/RV in one sample, as mentioned above, with a mean fluorescence intensity (MFI) of 424 (cutoff threshold for EV/RV by RVP Fast of 300). This sample tested negative by the RVP Classic assay and could not be confirmed by gel-based nested RT-PCR (4). This finding suggests the detection of a false-positive result or a low viral load in the specimen for EV/RV. A further breakdown of the detection of individual targets and discordant analysis is presented in the following sections.

Performance of the RVP Fast assay for individual targets. Results obtained by a head-to-head comparison for targets detected by both assays are discussed in the following sections, along with detailed analyses of each of the respiratory targets. The RVP Classic assay was used as the "gold standard" for the detection of respiratory viral targets for the calculation of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the RVP Fast assay, and these results, including kappa values, are provided in Table 3.

Influenza A virus. A total of 63 IFVA-positive samples were included in the comparison; all samples were positive by the RVP Classic assay, and two of these samples were negative for IFVA by the RVP Fast assay. The negative samples gave equivocal MFIs of 224 and 214 by the RVP Classic assay and C_T values of 38.5 and 34.6, respectively, by the real-time RT-PCR confirmatory assay (18). The sensitivity and specificity for IFVA of the RVP Fast assay were 96.83% and 100%, respectively, as indicated in Table 3. A total of 6 samples that tested positive for IFVA had coinfecting viruses: EV/RV ($n = 4$), hBoV ($n = 1$), and ADV ($n = 1$).

The 63 IFVA-positive samples included 22 samples of the pandemic (H1N1) 2009 subtype, 21 of the seasonal H1 subtype, and 20 of the seasonal H3 subtype, as determined by real-time RT-PCR assays (18, 21). The pandemic (H1N1) 2009 subtype could be detected as being IFVA positive but was not subtyped by the RVP Classic assay and provided an untypeable "UtD" result with the RVP Fast assay. All samples that were positive for IFVA but could not be typed ($n = 22$) gave concordant results by the two assays and were of the pandemic (H1N1) 2009 subtype, as confirmed by real-time RT-PCR (21). A total of 21 samples positive for IFVA of the seasonal H1 subtype provided 100% concordant results. Eighteen samples of the seasonal H3 subtype were detected by the RVP Classic assay, and two additional IFVA-positive samples were typed as being H3 positive by the RVP Fast assay. The two discordant samples had MFI values of 1,040 and 2,867 by the RVP Fast assay and C_T values of 34.0 and 32.5, respectively, by the subtyping assays. Thus, discordant IFVA and subtyping results were few and likely due to a low viral load.

Influenza B virus. A total of 46 IFVB-positive samples were included in this study. Of these, only 19 samples tested positive for IFVB by the RVP Fast assay, resulting in 27 discordant results. All IFVB-positive samples were confirmed by a previously reported real-time RT-PCR assay (18). The C_T values for the concordant samples ranged from 10.36 to 29.48, with a median of 21.9, and the C_T values for the discordant samples ranged from 22.43 to 34.84, with a median of 29.18, by real-time RT-PCR. Of the discordant samples ($n = 27$), 17 samples (62.96%) had a C_T value of less than 30, and 10 samples (37.04%) had a C_T value of greater than 30. A subset of 11 samples were reextracted using the manufacturer-recom-

TABLE 3. Performance of the RVP Fast assay for individual targets[a]

Virus target	No. of specimens tested	No. of coinfected specimens	No. of specimens with RVP Classic/RVP Fast results of:				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa
			+/+	+/−	−/+	−/−					
hCoV 299E	17	6	14	3	0	317	82.38	100	100	99.06	0.899
hCoV HKU1	14	5	14	0	0	320	100	100	100	100	1.000
hCoV NL63	12	2	11	0	1	322	100	99.69	91.67	100	0.955
hCoV OC43	15	5	13	0	2	319	100	99.38	86.67	100	0.926
PIV1	15	5	14	1	0	319	93.33	100	100	99.69	0.964
PIV2	11	2	7	4	0	323	63.64	100	100	98.78	0.772
PIV3	13	3	13	0	0	321	100	100	100	100	1.000
PIV4	12	2	12	0	0	300	100	100	100	100	1.000
RSVA	16	5	15	0	1	318	100	99.69	93.75	100	0.966
RSVB	22	12	21	1	0	312	95.45	100	100	99.68	0.975
hMPV	30	11	28	2	0	304	93.33	100	100	99.35	0.962
ADV	29	12	27	1	1	305	96.43	99.67	96.43	99.67	0.961
IFVA	63	6	61	2	0	271	96.83	100	100	99.27	0.980
IFVA-pH1N1	22	4	22	0	0	312	100	100	100	100	1.000
IFVA-H1	21	2	21	0	0	313	100	100	100	100	1.000
IFVA-H3	20	0	18	0	2	314	100	99.37	90	100	0.944
IFVB	46	2	19	27	0	288	41.3	100	100	91.43	0.548
EV/RV	40	30	34	0	6	294	100	98.0	85.00	100	0.909

[a] The numbers of positive and negative specimens detected by both testing methods are shown. This table shows the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for every target using the RVP Classic assay as the gold standard for comparison. It also shows the kappa coefficient and kappa statistics, including 95% confidence intervals, comparing the RVP Classic and RVP Fast assays. Targets included are influenza A virus (IFVA); IFVA subtype H1 (IFVA-H1); IFVA subtype H3 (IFVA-H3); the IFVA pandemic (H1N1) 2009 subtype (IFVA-pH1N1); influenza B virus (IFVB); respiratory syncytial virus A (RSVA); RSVB; human coronaviruses (hCoVs) NL63, 229E, OC43, and HKU1; parainfluenza viruses (PIVs) 1 to 4; human metapneumovirus (hMPV); picornaviruses (including enteroviruses [EVs] and rhinoviruses [RVs]); and adenovirus (ADV). A total of seven samples positive for hBoV were detected by the RVP Fast assay; the RVP Classic assay does not test for the presence of hBoV and thus is not included in the table.

mended protocol with a 200-μl specimen input volume and a 55-μl output volume and using our current protocol with a 200-μl specimen input volume and a 110-μl output volume. Similar results were obtained by using both extracts, confirming that the loss in sensitivity for the detection of IFVB was not the result of a change in the extraction volume.

To further understand the decreased sensitivity for IFVB, a recombinant plasmid containing the hemagglutinin (HA) gene of IFVB was tested by the RVP Fast assay to determine the region of detection. This plasmid tested positive by the RVP Fast assay, suggesting that the assay targets the HA region of the genome. The full-length HA gene from representative IFVB-positive specimens that were part of this study was sequenced. The phylogenetic relatedness between these HA sequences and prototype strains for the Victoria (B/Brisbane/60/2008 [GenBank accession number 766840.1]) and Yamagata (B/Florida/4/2006 [GenBank accession number CY033876.1]) lineages is shown in Fig. 1. Figure 1 indicates the sample number followed by the C_T value, which is indicative of the viral load in the sample. Of the IFVB-positive samples that were sequenced, 3 samples were collected prior to August 2008; 2 of these samples were of the Yamagata lineage (se-

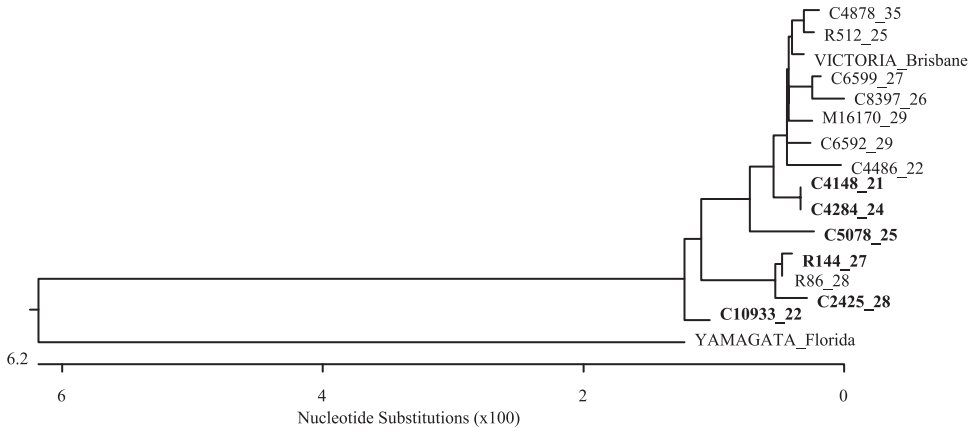

FIG. 1. Phylogenetic tree showing the relatedness between IFVBs detected in this study based on the hemagglutinin gene. The length of each pair of branches represents the distance between sequence pairs. The scale below the tree indicates the number of nucleotide substitutions, and the units show the numbers of substitution events. The sample number is shown, followed by the C_T value, which is indicative of viral load. The prototype strains for the Victoria (B/Brisbane/60/2008 [GenBank accession number 766840.1]) and Yamagata (B/Florida/4/2006 [GenBank accession number CY033876.1]) lineages are also included as references. The samples detected to be positive by the RVP Fast assay are shown in boldface type.

quence not shown), and 1 was of the Victoria lineage. All sequenced samples collected after August 2008 were of the Victoria lineage. The samples detected to be positive by the RVP Fast assay are shown in boldface type in Fig. 1. Based on sequence variation and the clustering of samples that were not detected by the RVP Fast assay in Fig. 1, it can be speculated that there may be changes in the primer or probe binding regions, resulting in a reduced sensitivity for the detection of IFVB. In addition, serial dilutions of extracts from positive specimens from 2008, 2009, and 2010 were tested by the RVP Classic and RVP Fast assays (Table 1). Endpoint detections were comparable between the two assays for the samples from 2008 and 2009; however, the RVP Classic assay was more sensitive for the detection of the IFVB-positive specimens from 2010 tested here (Table 1).

Of the IFVB-positive specimens, two samples were additionally positive for other respiratory viruses: one for EV/RV and one for PIV3. The sensitivity for IFVB was low, at 41.3% by the RVP Fast assay, and it cannot be used as the primary assay for the detection of IFVB.

Parainfluenza viruses. Of the 15 PIV1-positive samples, 1 sample tested negative by the RVP Fast assay; the discordant sample had an MFI of 1,429 by the RVP Classic assay and a C_T of 32 by a previously reported in-house-validated real-time RT-PCR assay (23). Five of these samples showed the presence of coinfecting viruses: EV/RV ($n = 2$), hCoV HKU1 ($n = 2$), and hCoV 229E ($n = 1$). A total of 11 PIV2-positive samples were tested, 4 of which tested negative by the RVP Fast assay. The MFI values for these four samples ranged from 297 to 2,374, with a median value of 1,139, by the RVP Classic assay, and the C_T values ranged from 29 to 32.5, with a median of 32.34. The RVP Fast assay thus showed a reduced sensitivity for PIV2 at 63.64% with 100% specificity. Two of the PIV2-positive samples indicated the presence of coinfecting viruses: hCoV HKU1 and RSVA. A total of 13 PIV3 and 12 PIV4 samples were included in the study, and no discordant results were detected. Of the 13 PIV3-positive samples, 3 samples showed the presence of coinfecting viruses: IFVB, EV/RV, hBoV, and RSVA. Of the 12 PIV4-positive samples, 2 samples showed the presence of coinfecting viruses: EV/RV and hCoV OC43.

Respiratory syncytial virus. The RVP Fast assay detects the presence of RSV1 and RSV2. For our experiments we have used retrospectively identified RSVA and RSVB samples with the RVP Classic assay. For the samples tested here, the RSVA and RSVB samples detected by the RVP Classic assay were identified as being RSV1 and RSV2 by the RVP Fast assay, respectively. A total of 38 RSV-positive samples, 16 RSVA and 22 RSVB samples, were tested. For RSVA, one sample tested positive by the RVP Fast assay but negative by the RVP Classic assay; this sample had an MFI of 298 by the RVP Fast assay and a C_T value of 37.5 by a real-time RT-PCR assay (22). One sample positive for RSVB by the RVP Classic assay (MFI = 610) was negative by the RVP Fast assay; this sample had a C_T of 35.4 by RT-PCR (22). Thus, discordant specimens for RSVA and RSVB had a low viral load. A total of 5 RSVA-positive samples showed the presence of coinfecting viruses, 1 each with ADV and PIV2, 2 with EV/RV, and 1 which showed the presence of PIV3 and EV/RV in addition to RSVA. A large number of samples positive for RSVB ($n = 12$) showed the presence of coinfecting viruses, including ADV, EV/RV, hMPV, hCoVs 229E and OC43, and PIV3.

Human metapneumovirus. There were a total of 30 hMPV-positive samples in the study. Of these, 28 were concordant and 2 were discordant between the RVP Classic and RVP Fast assays. The discordant samples had MFIs of 155 and 280 by the RVP Classic assay, with C_T values of 37 and 37.5, respectively, by real-time RT-PCR (19). Eleven of the 30 hMPV-positive samples showed the presence of coinfecting viruses, including EV/RV ($n = 3$), RSVB ($n = 3$), hCoVs HKU1 and NL63 and ADV ($n = 1$ each), and RSVB and EV/RV coinfection ($n = 2$).

Adenovirus. A total of 29 ADV-positive samples were included; of these, 1 sample each tested negative by the RVP Fast and RVP Classic assays, and both samples were positive by the confirmatory PCR assay. The sample that tested negative by the RVP Fast assay had an MFI value of 1,581 by the RVP Classic assay and a C_T of 27 by real-time PCR (24). This sample showed the presence of a high viral load of hBoV, suggesting competitive inhibition for the detection of ADV. The sample that tested negative by the RVP Classic assay had a C_T of 32 and an MFI of 424 by the RVP Fast assay. A total of 12 samples were also positive for additional coinfecting viruses: EV/RV ($n = 4$), RSV ($n = 2$), hBoV ($n = 2$), and IFVA, hCoV 229E, and hMPV ($n = 1$ each). One sample tested positive for EV/RV and hBoV.

Human coronaviruses. A total of 17 hCoV 299E-positive samples were tested, 14 samples of which were positive by both assays and 3 of which were negative by the RVP Fast assay. The MFI values for these samples were 610, 845, and 980 by the RVP Classic assay, and the C_T values were 34.4, 34.5, and 33.5, respectively. This resulted in a sensitivity and a specificity of 82.38% and 100%, respectively, for the detection of hCoV 299E by the RVP Fast assay. Fourteen hCoV HKU1-positive samples were tested by both methods, and 100% concordance was observed for detection. A total of 11 hCoV NL63-positive samples were included in the data set; all these samples tested positive by the RVP Classic and RVP Fast assays. The RVP Fast assay detected the presence of an additional positive sample with an MFI of 1,028 and a C_T of 32 by real-time RT-PCR (in-house [our unpublished data]). The sensitivity and specificity for the detection of hCoV NL63 were thus 100% and 99.69%, respectively. A total of 13 concordant hCoV OC43-positive samples were detected in the data set; 2 additional positive samples were detected by the RVP Fast assay, with MFI values of 973 and 3,599. Both samples were confirmed as being positive by real-time RT-PCR, with C_T values of 33.77 and 36. The RVP Fast assay thus had a sensitivity of 100% and a specificity of 99.38% for the detection of hCoV OC43. A large proportion of samples positive for human coronaviruses were also positive for other coinfecting viruses, including PIVs 1 to 4 ($n = 7$), RSV ($n = 5$), EV/RV ($n = 4$), ADV ($n = 1$), and hMPV ($n = 2$); OC43 and NL63 were detected as coinfections in two specimens.

Enteroviruses and rhinoviruses. Of the 334 samples included in this study, 34 specimens had tested positive for picornaviruses. The detection of the EV/RV target in these samples was concordant by the two assays. The RVP Fast assay additionally detected the presence of EV/RV in 6 samples, 5 of which were coinfections. The EV/RV target was detected in

one specimen, but its presence could not be confirmed by the gel-based nested RT-PCR (4) used to confirm all the discordant results for picornaviruses. The majority of the samples that were positive for the EV/RV target also showed the presence of coinfecting viruses ($n = 30$). The RVP Fast assay thus had superior sensitivity for the detection of EV/RV compared to that of the RVP Classic assay.

Human bocavirus. Human bocavirus testing is not included in our current testing algorithm. Of the 334 study samples tested, the RVP Fast assay detected the presence of bocavirus in 7 samples. All positive samples were tested by an in-house real-time RT-PCR assay for bocavirus (our unpublished data), with concordant results. All the 7 bocavirus-positive samples had another virus detected by the RVP Fast assay; these included ADV ($n = 2$), PIV3 ($n = 1$), IFVA ($n = 1$), and EV/RV ($n = 3$), and one sample showed the presence of RSVB, EV/RV, and ADV.

Internal controls. Of the total specimens tested ($n = 334$), 1 sample (0.30%) failed to give a valid result for MS2 by the RVP Fast assay, and 4 specimens (1.20%) failed to give a valid result by the RVP Classic assay. No failures were observed for the lambda external positive control, suggesting that the assays for the detection of the internal extraction and external PCR controls are reliable.

Performance of the RVP Fast assay for detection of coinfections. A total of 52 samples with coinfections were detected by the RVP Fast assay; of these, 43, 8, and 1 specimens were positive for 2, 3, and 4 viruses, respectively. Fewer samples ($n = 41$) showed the presence of coinfecting viruses by the RVP Classic assay, including 34 and 7 specimens that were positive for 2 and 3 viruses, respectively. The RVP Fast assay detected 13 more specimens with coinfections than did the RVP Classic assay; targets missed by the RVP Classic assay were hBoV ($n = 5$), EV/RV ($n = 5$), hCoV OC43 ($n = 2$), and ADV ($n = 1$). The RVP Classic assay detected the presence of coinfections in two samples; IFVB and PIV2 were the targets missed by the RVP Fast assay. One sample was positive for ADV and hBoV; ADV was not detected by the RVP Fast assay, and hBoV was not detected by the RVP Classic assay. The RVP Fast assay detected the presence of a third coinfecting virus in 2 samples, and the discordant targets were NL63 and RSVA. The RVP Fast assay also detected the presence of a fourth coinfecting hBoV in one sample. The majority of the discordant samples with multiple viruses were the result of the enhanced sensitivity of the RVP Fast assay for EV/RV and the detection of bocavirus.

DISCUSSION

In this report we have compared the older version of the xTAG RVP assay (RVP Classic) to the xTAG RVP Fast assay. The RVP Fast assay detects 19 respiratory viral targets (and differentiates between 18); this provides economical and timely results for the diagnosis of respiratory tract infections. The Luminex technology is flexible, and the assay can be altered in the future to accommodate more probes for the detection of novel viruses or new lineages for the subtyping of viruses, if necessary. In general, the use of this technology was previously reported to increase the rate of diagnosis for respiratory tract infections (7, 10, 14, 25). The RVP assay has been designed to include an RNA bacteriophage, MS2, as an extracted control for all specimens. MS2 is coamplified with the target and is a valuable internal extraction and amplification control for the monitoring of extraction and inhibition issues. Bacteriophage lambda is included as a DNA control in every run and controls for the amplification and detection steps of the assay. No significant inhibition of MS2 or failures to detect lambda were noted, suggesting that these controls are robust and reliable.

A comparison of the detection of IFVA, ADV, RSV, hMPV, PIV1, PIV3, PIV4, EV/RV, and hCoVs OC43, HKU1, and NL63 and subtyping of IFVA shows that the RVP Fast assay meets our current needs for diagnostic sensitivity and specificity for these targets. All the positive specimens for these targets missed by the RVP Fast assay were weakly positive by the RVP Classic and confirmatory PCR/RT-PCR assays.

The RVP Fast assay showed a reduced sensitivity for the detection of IFVB compared to those of the RVP Classic assay and real-time RT-PCR. The sensitivity for the detection of IFVB was reported to be 93.10% compared to a direct fluorescent-antibody assay (DFA) and/or viral culture based on the product insert. Gadsby et al. (7) previously reported a sensitivity of 100% for the detection of IFVB; however, only four positive samples were tested in that study. As mentioned in Results, sequence variation is present in the hemagglutinin gene detection region of IFVB, and it is possible that base pair mutations in the primer or probe binding regions have altered target sensitivities. In our diagnostic laboratory, screening for IFVB will be performed by using real-time RT-PCR (18). This study shows a drop in sensitivity for the detection of PIV2 and hCoV 299E compared with that of the RVP Classic assay. The significance of the loss of sensitivity for PIV2 may be ameliorated by the fact that PIV2 has the lowest prevalence of the PIVs tested, and the detection of PIV2 is greatly enhanced by nucleic acid amplification tests (NATs) compared to traditional DFA-based methods (5). We also believe that a slightly lower sensitivity for the detection of hCoV 229E is acceptable since these viruses have not been tested historically, and no specific management protocols are instituted when a positive specimen is detected; however, surveillance data can be impacted.

Preliminary studies show that the use of the RVP Fast assay will result in cost savings and a reduced hands-on time of approximately 1.5 h, in addition to an improved turnaround time as a result of the shorter protocol. The technology is flexible, and the assay is sensitive, specific, and high throughput, allowing the use of 96-well plates for batch testing as appropriate. Results for 19 viral targets are available within 8 h of receipt of specimens. The RVP assay is comprehensive and includes all the respiratory viral targets that are currently tested routinely for the diagnosis of acute respiratory tract infections.

The results of this study indicate that the RVP Fast assay may be a new tool in clinical laboratories for respiratory virus testing, especially in laboratories that are starting to implement molecular techniques. However, in laboratories utilizing the RVP Classic assay, there will be changes in viral prevalence for several targets due to the drop in the sensitivity of the RVP Fast assay. This may also have a negative impact on surveillance programs that previously utilized the RVP Classic assay as a tool for the monitoring of viral prevalences. Furthermore,

due to the poor sensitivity of this assay for IFVB detection, we suggest that IFVB detection should be performed by an alternate method until a further adaptation of the RVP Fast assay addresses the loss in sensitivity.

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